Biochemical and Biophysical Research Communications 422 (2012) 578–585

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Targeted *Casp8AP2* methylation increases drug resistance in mesenchymal stem cells and cancer cells

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ARTICLE INFO

Article history:
Received 6 May 2012
Available online 14 May 2012

Keywords:
DNA methylation
Epigenetic
Histone modification
Cell death
Chromatin immunoprecipitation

ABSTRACT

Casp8AP2 contains a FLASH functional domain and is critical for the formation of death complex and the relay of death signal into the cells. Genetic defects in *Casp8AP2* are associated with several diseases. A CpG island within the *Casp8AP2* promoter is differentially regulated during somatic stem cell differentiation, and aberrant DNA methylation within the *Casp8AP2* promoter has been reported in cancers. We hypothesized that abnormal DNA methylation of *Casp8AP2* promoter might contribute to prolonged cellular survival or drug resistance in cancer. The epigenetic state within the *Casp8AP2* promoter was then determined in different cancer cell lines and patient samples by methylation-specific PCR. Targeted *Casp8AP2* methylation within normal and tumor cells was performed to see whether methylation promoted drug resistance. We found differential *Casp8AP2* methylation among the normal and tumoral samples. Global demethylation in a platinum drug-resistant human gastric cancer cell line reversed *Casp8AP2* methylation and diminished drug resistance. Targeted methylation of the *Casp8AP2* promoter in somatic stem cells and cancer cells increased their resistance to drugs including platinum drugs. These data demonstrate that methylation within the *Casp8AP2* promoter correlates with the development of drug resistance and might serve as a biomarker and treatment target for drug resistance in cancer cells.

1. Introduction

Death signals appear to be relayed into cells through death signaling pathways [1–4], and disturbance of these pathways may contribute to the development of diseases like cancer [5–7] or drug resistance [8,9] during tumoral progression. *Casp8AP2* (NM_0011 37667) associates with Caspase 8 (CASP8, NM_033356) to form the death-inducing signaling complex (DISC) which regulates the programmed cell death and cell cycle/survival [10,11]. Fine-tuned *Casp8AP2* expression is required during embryogenesis [12] and abnormal expression of *Casp8AP2* is observed in the hippocampus of ischemic mice [6]. Altered *Casp8AP2* expression is also correlated with the prognosis and the development of minimal residual disease in childhood acute lymphoblastic leukemia [13–15]. In addition, *Casp8AP2* expression responds to different drugs differently [16]. These findings suggest that changes in *Casp8AP2* expression might lead to the changes in cell fate like cellular transformation or the development of drug resistance.

DNA methylation is an inherited form of epigenetic regulation that dominantly silences the affected gene [17–19]. Normal and abnormal environmental factors like growth factors or carcinogens are hypothesized to modulate methylation by generating cellular signals that are memorized as epigenetic marks that control cellular differentiation or the onset of diseases [20]. The cellular methylation differences created by these signaling pathways provide differential expression within cells of a population that could then be further selected into clonal populations of cancerous or drug-resistant cells. Recently, we have applied a targeted DNA methylation

Abbreviations: MSC, mesenchymal stem cell; 5-Aza, 5-aza-2′-deoxycytidine; H3K27me3, histone 3 lysine 27 trimethylation; H3K4me3, histone 3 lysine 4 trimethylation.

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http://dx.doi.org/10.1016/j.bbrc.2012.05.029

(TDM) methodology [21,22] to demonstrate that DNA methylation within critical loci is sufficient to promote somatic stem cell differentiation [23], and to transform a normal stem cell [22]. In this study, we use TDM to test the hypothesis that methylation within the *Casp8AP2* promoter can inhibit the cell death induced by anticancer drugs.

In this report, we determined the epigenetic state of the *Casp8AP2* promoter in several different cancer cell lines and detected methylation differences between brain, breast and liver cancer samples from patients. To demonstrate that the expression of *Casp8AP2* is regulated by DNA methylation, we treated the oxaliplatin-resistant human gastric cancer cell subline S3 with methylation inhibitor, 5-aza-2′-deoxycytidine (5-Aza) [24,25] and found elevated *Casp8AP2* expression. Restored expression of *Casp8AP2* also correlated with the reversion of drug resistance. Finally, we used a targeted DNA methylation approach to methylate the *Casp8AP2* promoter in normal (mesenchymal stem cells, MSC) and breast cancer cell (MDA-MB-231) and found that *Casp8AP2* methylation

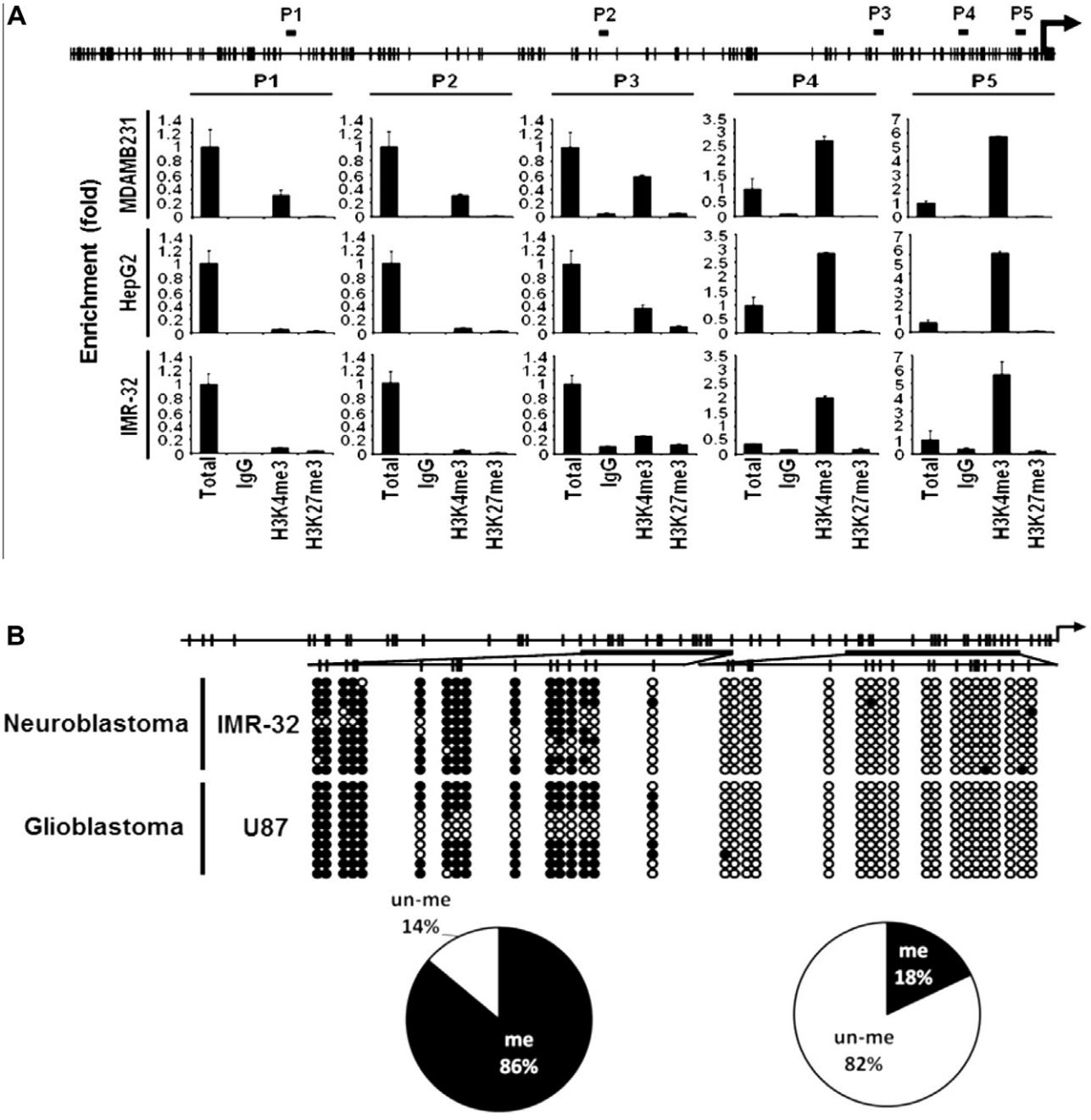

Fig. 1. Epigenetic state of *Casp8AP2* promoter. (A) Chromatin conformation of *Casp8AP2* promoter in designated cell lines was detected by chromatin immunoprecipitation PCR (ChIP-PCR). The physical map of *Casp8AP2* promoter is illustrated on top and the vertical bars indicate the CpG sites. The ChIP-PCR amplification/detection regions are marked by horizontal bars (P1-P5). The arrow indicates the transcriptional start site (TSS). Using semi-quantitative real time PCR, the enrichment of H3K4me3 and H3K27me3 was calculated in comparison with total input. IgG-immunoprecipitation was used as a negative control. (B) Bisulfite sequencing of the *Casp8AP2* promoter. Bisulfite sequencing was used to detect the methylation state of the *Casp8AP2* promoter in IMR-32 and U87 cells. The physical map of the *Casp8AP2* promoter is illustrated on top and the CpG sites are marked by vertical bars. Filled and open circles indicate methylated and unmethylated CpG sites, respectively. Two promoter regions were sequenced and the overall methylation states were calculated and are shown in the pie chart.

reduced sensitivity of the MSC and MDA-MB-231 cells to several drugs.

2. Material and methods

2.1. MSC and cell lines culture

Human MSC isolation and culture were performed as described previously [23,26]. Cancer cell lines were cultured in either L-15 (MDA-MB-231 cells) or Dulbecco's modified Eagle medium (DMEM) (HepG2, U87, and IMR-32 cells). The oxaliplatin/cisplatin-resistance human gastric adenocarcinoma TSGH cell subline, S3, was isolated and maintained as previously reported [27]. All culture media was supplemented with 10% fetal bovine serum (Invitrogen), 100 mg/ml penicillin/streptomycin (Invitrogen), and 2 mM L-glutamine (Invitrogen).

2.2. 5-Aza treatment

Cells were treated with 5 μM of 5-Aza or an equal final volume of DMSO (as solvent) for 5 consecutive days.

2.3. Targeted DNA methylation

Targeted DNA methylation was performed by three times of transfection of the denatured, methylated DNAs that are complementary to the promoter regions of the target genes. The *in vitro* methylation, labeling of the methylated DNAs and transfection of these DNAs are describe as followed:

2.4. In vitro DNA methylation

Both of the *Casp8AP2* and *ENSA* (as control) promoters were selected from human CpG island clones [28,30], PCR-amplified (T_m = 54 °C; Primers: hGMP1.1, 5′ CGGCCGCCTGCAGGTCTGACCATAA 3′; hGMP2.1, 5′ AACGCGTTGGGAGCTCTCCCATAA 3′) and purified. The target region for *Casp8AP2* promoter was indicated in Supplementary Fig. 2A (filled upper panel) and the aligned sequence is shown in the lower panel. The targeted sequence and alignment validation of *ENSA* is shown in Supplementary Fig. 2B. Then 4 μg of the two different PCR products were *in vitro* methylated by incubation with 20 Units of CpG methyltransferase (*Sss*I, New England Biolabs) at 37 °C for 4 h in the presence of 160 μM

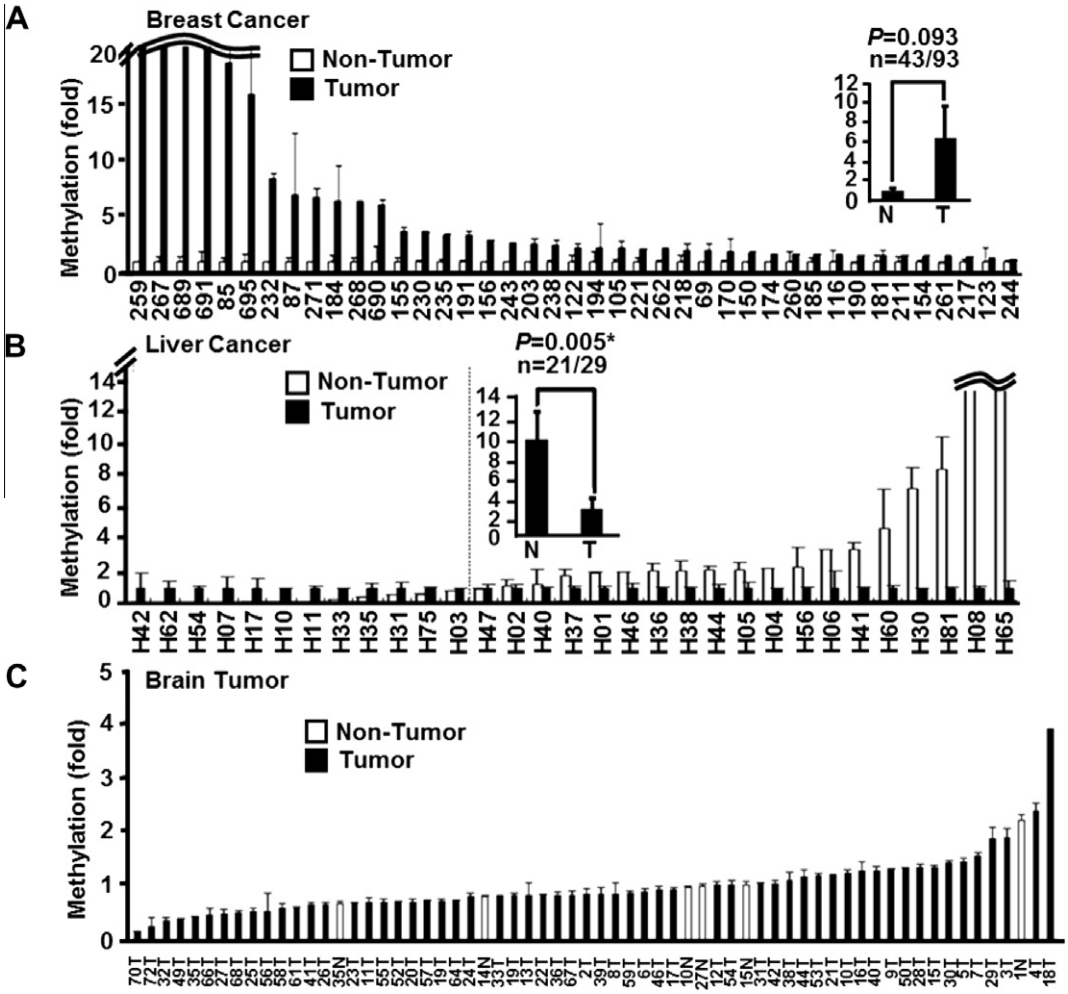

Fig. 2. *Casp8AP2* methylation in patient samples. Semi-quantitative real-time methylation-specific PCR (qMSP) and bisulfite sequencing were used to detect the *Casp8AP2* methylation state in different cancer patient samples. (A) *Casp8AP2* methylation difference between paired adjacent normal (open bars) *versus* tumor (closed) from breast cancer patients. Forty-six of ninety-three tumor samples had hypermethylated *Casp8AP2* relative to normal adjacent tissue [methylation fold = (tumor methylation)/(normal methylation)]. (B) *Casp8AP2* methylation difference between paired adjacent normal (open bars) *versus* tumor (closed) from liver cancer patients. Seventeen out of twenty-nine tumor samples had hypomethylated *Casp8AP2* relative to normal adjacent tissue [methylation fold=(normal methylation)/(tumoral methylation)]. (C) *Casp8AP2* methylation states from different brain tumors. *Casp8AP2* methylation readings were normalized relative to the *Col2A1* readings in the same sample. (D) Bisulfite sequencing results from different paired brain tumor *versus* non-tumor (normal) part. The physical map and detection regions are as shown in Fig. 1B. The filled and open circles indicate the methylated and unmethylated CpG sites respectively.

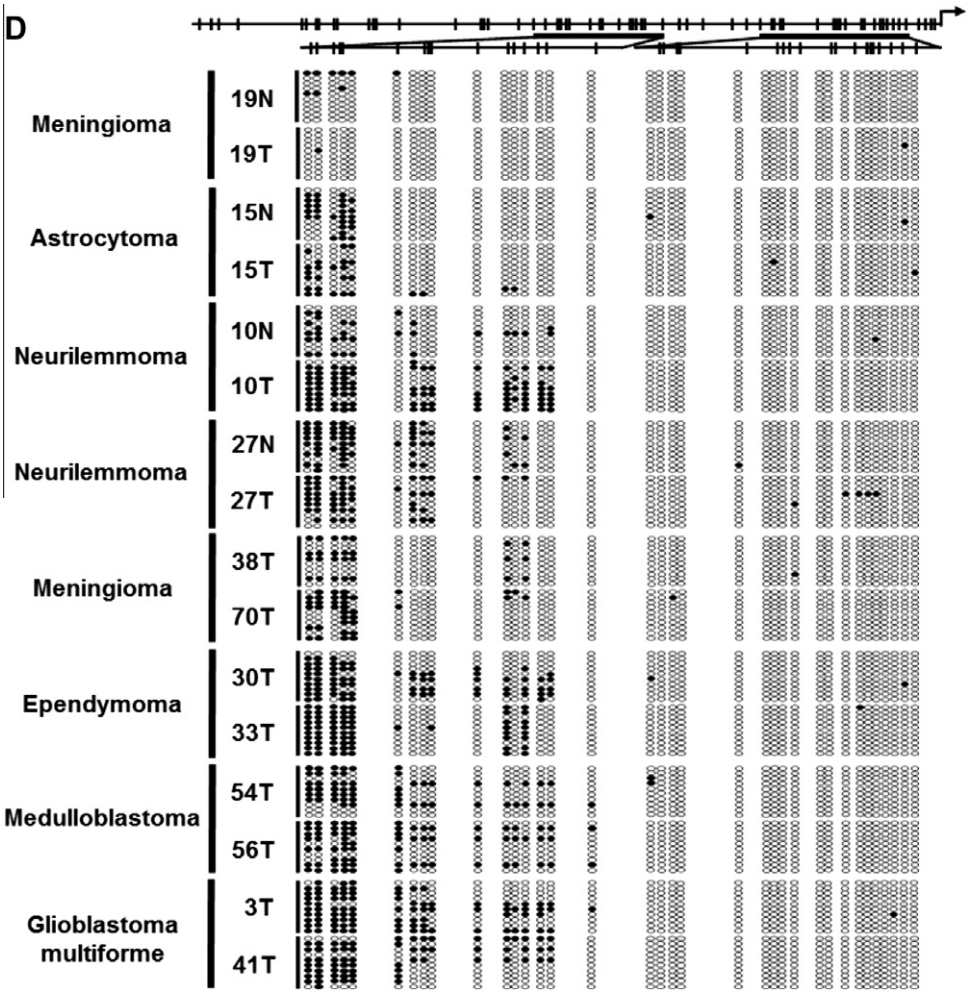

Fig. 2 (*continued*)

S-adenosylmethionine to induce methylation, followed by heating at 65 °C fro 5 min. Complete methylation was confirmed by demonstrating resistance to methylation-sensitive restriction enzymes (*Bst*UI) (Supplementary Fig. 1).

2.5. Cy5 labeling of the Casp8AP2 promoter fragment

Casp8AP2 DNA was labeled with *Label*IT tracker Reagents (Mirus) according to the manufacturer's instructions.

2.6. Transfection

Methylated PCR products (0.4 μg/well in 6-well plate) were denatured at 95 °C for 5 min, chilled on ice for 2 min and then transfected into 5×10^5 cells/well in 6-well plate using DMRIE-C (Invitrogen) according to the manufacturer's instructions. Unmethylated PCR products were transfected as mock control. Cells were transfected three times at day 1, 3, and 5.

2.7. Chromatin Immunoprecipitation (ChIP) assay

ChIP-PCR and semi-quantitative ChIP-PCR (qChIP-PCR) were performed as described previously [23,28]. Antibodies were purchased from Upstate.

2.8. Semi-quantitative real-time methylation-specific PCR (qMSP)

qMSP assays were performed according to the protocol described in Yan et al. [29]. Briefly, bisulfite converted genomic DNAs (0.5 μg) were subject to real-time PCR with methylation specific primers (Supplementary Table 1). qMSP reactions were performed using the SYBR Green I PCR Kit (Toyobo) in an iQ5 Real-Time PCR instrument (Bio-Rad). Melting analysis was performed for each PCR reaction to insure a specific amplicon was generated. *Col2A1* (NM_033150) amplified bisulfite-converted DNA was serially diluted and used to generate a standard curve and as a loading control. Methylation percentage was calculated as: [Means of target gene]/[Means of *Col2A1*]; fold change was calculated as: [TDM methylation percentage]/[Mock methylation percentage].

2.9. Bisulfite sequencing

Genomic DNA (0.5 μg) was bisulfite-converted, PCR-amplified, cloned, and sequenced as described by Yan et al. [29] using primers listed in Supplementary Table 1.

2.10. Cloning of the human Casp8AP2 promoter

Primer sequences for human *Casp8AP2* promoter are listed in Supplementary Table 1. Genomic DNA purified from human mesenchymal stem cells (MSCs) served as a template for PCR. Purified

PCR products were ligated into the pyT&A cloning vector (Yeastern Biotech) according to the manufacturer's protocol. Inserts were confirmed by restriction digestion and sequencing.

2.11. Semi-quantitative RT-PCR, qRT-PCR

Total RNA isolation, first-strand cDNA synthesis, and detection of the transcripts were performed as described [30]. Briefly, total RNA (2 μg) was reverse transcribed using the SuperScript II reverse transcriptase (Invitrogen). qRT-PCR was then performed using the SYBR Green I PCR Kit (Toyobo) in an iQ5 Real-Time PCR instrument. A serial dilution of *GADPH* (NM_002046) amplified cDNA was used to generate standard curve and *GAPDH* was used as loading control. The primers are listed in Supplementary Table 1.

2.12. Cell survival (MTT) assay

Cells were plated in 96-well plates at approximately 5×10^4 cells/well and allowed to attach. The cells were then treated with designated concentrations of drugs and incubated at 37 °C for overnight. Twenty microliters of 5 mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) solution (Sigma) was added to each well and incubated at 37 °C for 5 h. The reaction was terminated by adding 200 μl of DMSO, and absorbance was measured at 595 nm.

2.13. Human subjects

Isolation and characterization of human MSCs were conducted under IRB regulations of the Chang Gung Memorial Hospital, Chiayi, Taiwan. Tumor samples were collected from Changhua Christian hospital under IRB regulation.

3. Results

3.1. Epigenetic state of the Casp8AP2 promoter in cancer cell lines

Casp8AP2 is part of the death complex in cells and its function is essential for cell death. *Casp8AP2* is thought to be actively transcribed in the majority of cells subjected to anti-cancer drug treatment. To assess the methylation status of *Casp8AP2*, we initially used ChIP to detect the chromatin conformation of the *Casp8AP2* promoter. We found that the active chromatin conformation, trimethylated histone H3 at lysine 4 (H3K4me3) [31], was enriched near the transcriptional start site (TSS) of *Casp8AP2* in MDA-MB-231 (breast cancer), HepG2 (liver cancer) and IMR-32 (neuroblastoma) cell lines (Fig. 1A). There was no detectable binding of a negative

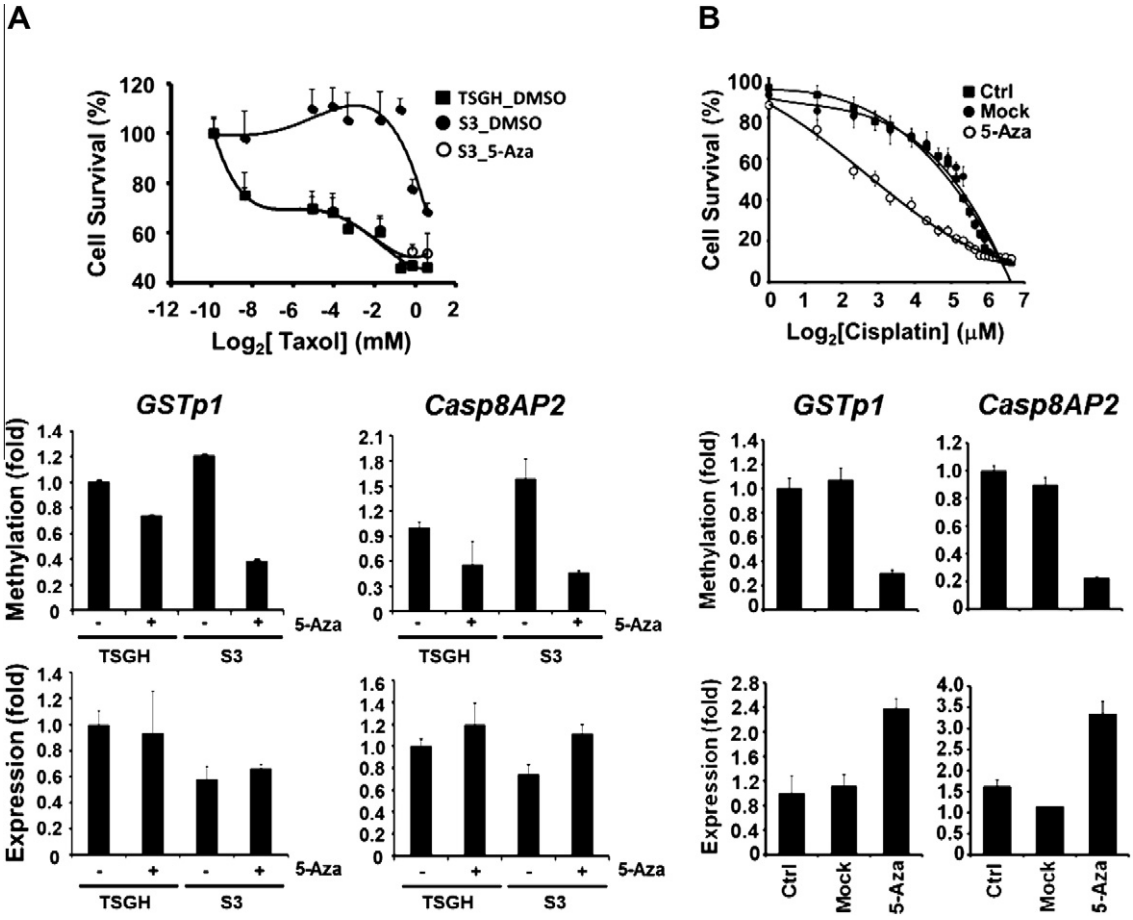

Fig. 3. 5-Aza treatment reactivates the methylation-silenced *Casp8AP2* and reverses the drug resistance associated with *Casp8AP2* methylation. (A) Dosage-dependent cell survival of gastric cancer cells. Taxol-resistant S3 gastric cancer cells were treated with 5-Aza or mock-treated with DMSO. The taxol-sensitive S3-parental gastric cancer TSGH cells were mock-treated with DMSO and served as a negative control. Cell survival was determined by MTT assay after challenge with different concentrations of taxol (upper). The methylation of *GSTp1* and *Casp8AP2* in S3 was reduced after treatment with 5-Aza (center panels), and their expression was restored (bottom). (B) Dosage-dependent cell survival of ovarian cancer cells. Cisplatin-resistant CP70 ovarian cancer cells were treated with 5-Aza or mock-treated with DMSO. Untreated CP70 was used as a control (Ctrl). MTT assay was utilized to detect the cell survival after challenge with different concentrations of Cisplatin (upper). The methylation of *GSTp1* and *Casp8AP2* in CP70 cells was reduced after treatment with 5-Aza (center panels), and their expression was restored (bottom).

chromatin conformation, trimethylated histone H3 at lysine 27 (H3K27me3) [32]. In agreement with the ChIP assay data, bisulfite sequencing detected no significant DNA methylation near the TSS in IMR-32 cells and U87 cells (glioblastoma), although there was significant DNA methylation upstream of the *Casp8AP2* promoter in both cell lines (Fig. 1B), 86% *versus* 18% methylation). A putative boundary between the methylated and unmethylated regions was identified (Fig. 2B, dashed line).

3.2. Abnormal Casp8AP2 methylation within patient samples

To determine whether dysregulation of DNA methylation within the *Casp8AP2* promoter occurs *in vivo*, qMSP and bisulfite sequencing were used to detect the *Casp8AP2* methylation state within cancer specimens (Fig. 2). We found that, relative to their paired adjacent normal samples, 49% of breast cancers samples ($n = 93$ cases) had significant hypermethylation of the *Casp8AP2* promoter (Fig. 2A), whereas 59% of the liver cancers ($n = 29$ cases) had significant hypomethylation (Fig. 2B). These data indicate that misregulation of DNA methylation might occur during tumorigenesis and the occurrence might be tissue or individual specific. Dysregulated DNA methylation of the *Casp8AP2* promoter was also detected in brain tumors (Fig. 2C). Using bisulfite sequencing, we noticed that the mal-regulated methylation rarely spread across the putative boundary between the methylated and unmethylated regions in the paired brain tumor samples (Fig. 2D).

3.3. Global demethylation reduced Casp8AP2 methylation and cancer drug resistance

To determine whether there is a correlation between increased *Casp8AP2* methylation and the development of drug resistance, we first treated drug resistant cancer cells with 5-Aza to demethylate globally and assayed drug sensitivity. After treatment with 5-Aza for 5 days, the drug resistant gastric cancer cell line S3 [27] became sensitive to Taxol just as the parental sensitive gastric cancer cells, TSGH, did [27] (Fig. 3A). The methylation of *GSTp1* [33,34] and *Casp8AP2* was reduced after the 5-Aza treatment, and the expression of these two genes was up-regulated accordingly (Fig. 3C). The correlated up-regulation of gene expression after treatment with 5-Aza indicates that methylation within the promoter regulates transcription of the corresponsive genes. Treatment with 5-Aza also repressed the drug resistance of CP70 [30], an ovarian cancer cell line (Fig. 3B). The methylation of *GSTp1* and *Casp8AP2* in CP70 cells were repressed after 5-Aza treatment, and the gene expression of *GSTp1* and *Casp8AP2* were correspondingly restored in CP70 cells (Fig. 3D).

3.4. Targeted Casp8AP2 methylation increased drug resistance in normal and tumor cells

To directly demonstrate a role for *Casp8AP2* methylation in induced drug resistance in normal and tumor cells, we used the

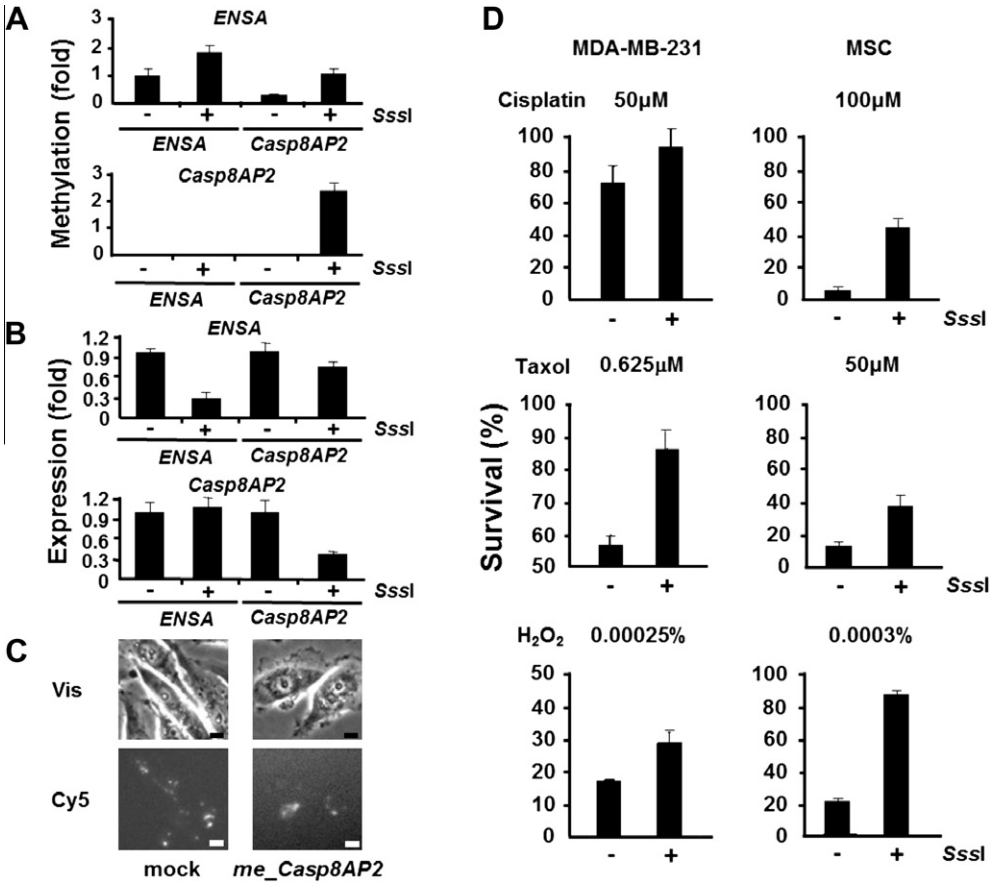

Fig. 4. Targeted *Casp8AP2* methylation increases drug resistance. (A) Targeted *Casp8AP2* methylation. *In vitro* methylated (*Sss*I treated) *ENSA* or *Casp8AP2* induced endogenous *ENSA* (upper) or *Casp8AP2* (lower) methylation, respectively, but did not alter methylation of the other locus. (B) Targeted *Casp8AP2* methylation suppressed *Casp8AP2* gene expression. *In vitro* methylated *ENSA* or *Casp8AP2* suppressed *ENSA* (upper) or *Casp8AP2* (lower) expression, respectively, as detected by qRT-PCR. (C) Tracking the transfected, labeled DNAs. *In vitro* methylated or unmethylated DNAs were Cy5-labeled and used for transfection. Transfection of labeled DNAs was tracked by fluorescence microscopy. The bar indicates 3.0 μm in length. (D) Increased cell survival after targeted *Casp8AP2* methylation. MDA-MB-231 cells and MSC were transfected with *in vitro* methylated or unmethylated *Casp8AP2* DNAs, and then challenged with different concentrations of Cisplatin, taxol and H_2O_2. Cell survival after drug treatment was detected by MTT assay.

TDM approach to methylate the *Casp8AP2* promoter in MSC (normal) and MDA-MB-231 (cancer) cells and then challenged those cells with anticancer drugs like cisplatin and taxol and with hydrogen peroxide (H_2O_2), a non-specific damaging agent. Targeted *Casp8AP2* methylation increased methylation at the *Casp8AP2* promoter, but not at the *ENSA* promoter, used as a control (Fig. 4A bottom and Supplementary Fig. 2). Targeted DNA methylation of the *ENSA* promoter increased *ENSA* methylation without affecting methylation of the *Casp8AP2* promoter (Fig. 4A, upper). The increased *ENSA* or *Casp8AP2* methylation decreased the expression of the corresponsive gene, respectively (Fig. 4B). Targeted DNA methylation was induced by the transfection of *in vitro* methylated DNAs that were tracked by Cy5 labeling as they entered the cell nuclei (Fig. 4C). MSC and MDA-MB-231 cells with targeted *Casp8AP2* methylation were then challenged by cisplatin, Taxol and H_2O_2. *Casp8AP2* methylation increased cell survival in every case (Fig. 4D and Supplementary Figs. 3 and 4).

4. Discussion

DNA methylation is a stable somatic inheritance that is associated with gene silencing and makes it a useful biomarker [35–37]. It has been hypothesized that environmental cues could be transmitted into cells and memorized as DNA methylation that further shapes gene expression patterns. These lineage-inherited expression differences could then be further selected, and potentially be associated with transformation or development of drug resistance [38–41]. Methylation affecting the expression of genes that comprise the death complex, like *Casp8AP2*, would be one way in which the relay of death signal could be suppressed and cell survival could be enhanced. Because DNA methylation is reversible and methylation inhibitors are now available [42,43], methylation-dependent drug resistance could potentially be reversed, making tumors more sensitive to anti-cancer drugs.

In this report, we identified dysregulation of *Casp8AP2* methylation in several cancers (Fig. 2), indicating a lineage-specific methylation change. Global demethylation in drug-resistance gastric and ovarian cancers suppressed *Casp8AP2* methylation, reactivated its expression, and correlated with the reversal of drug resistance (Fig. 3). Targeted methylation of the *Casp8AP2* promoter recapitulated the development of drug resistance in normal and tumor cells (Fig. 4). These data support the hypothesis that DNA methylation within the *Casp8AP2* locus is sufficient to reduce cellular sensitivity to drugs and suggest that the detection of mal-regulated *Casp8AP2* methylation might be a good reference for epigenetic therapy in the future.

Different levels of *Casp8AP2* methylation could be observed among different individuals consistent with individual differences in disease progression. Methylation of other loci might be important in certain lineages of tumoral evolution or in different individuals. Targeted DNA methylation of these loci would reveal their functional importance, and reveal which other loci might be useful as biomarkers for epigenetic factors in cell transformation and drug resistance.

Acknowledgments

YWL and KDL were supported by NSC Taiwan 98-2314-B-182-030-MY3. YWL, SHH and PYC are supported by NSC Taiwan (100-2320-B-194-001 and 100-2321-B-750-001). YWL and SHH are supported by CCU Taiwan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.029.

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